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Resistance of a Human Serum-Selected Human Immunodeficiency Virus Type 1 Escape Mutant to Neutralization by CD4 Binding Site Monoclonal Antibodies Is Conferred by a Single Amino Acid Change in gp120

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We have selected an HXB2 variant which can replicate in the presence of a neutralizing human serum. Sequencing of the gp120 region of the *env* gene from the variant and parental viruses identified a single amino acid substitution in the third conserved region of gp120 at residue 375 (AGT→AAT, Ser→Asn; designated 375 S/N). The escape mutant was found to be resistant to neutralization by soluble CD4 (sCD4) and four monoclonal antibodies (MAbs), 39.13g, 1.5e, G13, and 448, binding to epitopes overlapping that of the CD4 binding site (CD4 b.s.). Introduction of the 375 S/N mutation into HXB2 by site-directed mutagenesis confirmed that this mutation is responsible for the neutralization-resistant phenotype. Both sCD4 and three of the CD4 b.s. MAbs (39.13g, 1.5e, and G13) demonstrated reduced binding to the native 375 S/N mutant gp120. The ability to select for an escape variant resistant to multiple independent CD4 b.s. MAbs by a human serum confirms the reports that antibodies to the discontinuous CD4 b.s. are a major component of the group-specific neutralizing activity in human sera.

Neutralizing antibody appears to be an important component of the protective immune response against human immunodeficiency virus type 1 (HIV-1) infection (1, 4–6). Sera from many HIV-1-infected individuals are able to neutralize a broad spectrum of virus isolates, indicating the presence of conserved neutralization epitopes (28, 37). The induction of antibodies with broad neutralizing capacity is a primary goal of vaccine development strategies. Several reports have suggested that HIV-1 neutralizing activity is associated primarily with two regions of the gp120 envelope glycoprotein: the third hypervariable loop (V3) (8, 10, 29) and the CD4 binding site (CD4 b.s.) (11, 34). Generally, antibodies specific for V3 arise early after HIV infection and exhibit the ability to neutralize a limited number of HIV-1 isolates. However, a limited number of V3 monoclonal antibodies (MAbs) recognize conserved features of the loop and subsequently exhibit a broader neutralization profile (7, 10, 24). Later in the course of viral infection, antibodies capable of neutralizing a wider range of HIV-1 isolates are detectable (20, 28, 37). The appearance of such cross-neutralizing activity coincides with the detection of antibodies capable of blocking gp120-soluble CD4 (sCD4) interaction (9, 11, 18, 34). A number of neutralizing MAbs derived both from infected individuals (9, 12, 26, 30, 36) and from recombinant gp120-immunized rats (3) which block gp120-sCD4 binding have been identified, suggesting that such

antibodies may play an important role in the cross-neutralizing response (21).

The selection and characterization of neutralization-resistant escape mutants is one strategy for the identification of amino acid residues critical for the binding of neutralizing antibodies. We have selected escape variants which are resistant to both V3 MAbs and sCD4 (13, 15). To define the epitopes responsible for inducing cross-neutralizing antibodies in human sera further, we selected an escape variant that could replicate in the presence of a neutralizing human serum. Characterization of the neutralization sensitivity of the escape variant with a panel of MAbs mapping to the second variable loop (V2), V3, and the CD4 b.s. demonstrated that the variant was resistant to a number of MAbs mapping to the CD4 b.s., suggesting that antibodies specific for the CD4 b.s. in human sera are capable of cross-neutralizing a nonhomologous HIV-1 isolate (HXB2) and may be sufficient to select for neutralization-resistant escape mutants.

MATERIALS AND METHODS

Virus propagation and neutralization assays. (i) **Transfection of H9 cells and viral stock production.** H9 cells were washed twice in serum-free medium and resuspended at 10^6 /ml in RPMI. Ten micrograms of HXB2 plasmid was transfected into 2×10^6 H9 cells by the use of Lipofectin as instructed by the manufacturer (GIBCO BRL, Gaithersburg, Md.). The transfected H9 cultures were monitored for viral infection by the detection of p24 antigen (as described

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below) and by the appearance of multinucleated cells. Once the cells were producing virus (days 6 to 7 posttransfection), stocks of extracellular virus were made by mixing the infected cells with uninfected H9 cells in a 1:4 ratio. Forty-eight hours after the cocultivation, the supernatant was collected, aliquoted, and stored under liquid N₂. Infectivity determinations were made by performing 10-fold serial dilutions of virus (50 μ l) and incubation with 100 μ l of C8166 cells at 2×10^5 cells per ml in microtiter plates at 37°C for 7 days. Plates were scored for the presence of p24 antigen, and the 50% tissue culture infective dose (TCID₅₀) values were determined by the Karber formula.

(ii) **Neutralization assays.** HIV (10^3 TCID₅₀) in a volume of 40 μ l was incubated with 10 μ l of a dilution of antibody, serum, or sCD4 under test at 37°C for 1 h. The virus-antibody or virus-sCD4 mixture was then incubated with 100 μ l of C8166 cells at a concentration of 2×10^5 cells per ml per well in a microtiter plate in triplicate. Five days postinfection, the wells were scored for the presence of syncytia, and the extracellular supernatant was collected from the wells by centrifugation, inactivated with 1% Empigen, and assayed for soluble p24 antigen as described previously (17). The lowest concentration of antibody or sCD4 resulting either in a complete blocking of syncytium formation or in a >90% reduction in p24 antigen production was defined as the reciprocal neutralization titer.

(iii) **Comparative neutralization of variant and wt virus by human sera.** Human serum neutralization titers were obtained for both variant and wild-type (wt) viruses. The differences in the titers were compared by using a two-tail nonparametric test (Wilcoxon's rank sum) (see Fig. 7).

MAbs and human sera. The following antibodies were used for neutralization studies and/or envelope binding studies: rat MAbs 10/54, 10/36e, and 11/85b, specific for amino acids 311 to 321 of the HXB10 V3 loop (14); rat MAb 41.1, recognizing a conformation-dependent epitope within the V3 loop (14); rat MAb 10/76b, mapping to a linear epitope in the V2 loop (17a); 38.1a, specific for amino acids 425 to 441, involved in CD4 binding (3); 39.13g, a rat MAb specific for an epitope overlapping the CD4 b.s. (3, 19); and human MAbs 588, 559, 448, 728, 654, 729 (12, 19), 1.5e, 2.1h (9, 35), and G13 (30), binding to closely related epitopes overlapping the CD4 b.s.; and murine MAb L120, specific for CD4 (Medical Research Council [MRC] AIDS Directed Programme [ADP] Repository).

Sera were collected from 21 healthy CDC stage II and III seropositive males attending a sexually transmitted disease clinic as part of a longitudinal study of the natural history of HIV-1 infection (2). These individuals had not received antiretroviral therapy at the time of sampling.

Antibody and sCD4 binding to detergent-solubilized viral gp120. Virus-containing supernatants were inactivated with 1% Nonidet P-40 (NP-40), which does not irreversibly denature gp120/160 (23). The concentrations of gp120 present in the preparations were determined by a twin-site enzyme-linked immunosorbent assay (ELISA) as previously described (22, 23), using recombinant gp120 (rgp120; BH10 clone), expressed in and purified from CHO cells, as a reference standard. Briefly, detergent-solubilized gp120 from wt, variant, and 375 S/N (see Results for definition) site-directed mutant viral stocks was allowed to bind to the solid phase via the capture antibody D7324 (anti-C terminus; Aalto-Bioreagents, Dublin, Ireland) at an input concentration of 50 ng/ml. The abilities of MAbs and sCD4 to bind to the captured gp120 were assessed by using methods previously published (13, 19). In each case, the result is expressed as a

ratio of the optical density of the mutant protein to that of the wt protein and is termed the relative binding index.

MAb cross-competition analysis for rgp120 binding. The human CD4 b.s. MAbs and three control rat MAbs binding to the V2, V3, and fourth conserved (C4) regions of gp120 were tested for the ability to compete with iodinated preparations of MAb 39.13g for binding to D7324-bound rgp120. The unlabeled MAbs, at a concentration sufficient to saturate the gp120 (5.0 μ g/ml), were mixed with an equal volume of 39.13g, at a concentration resulting in half-maximal binding (0.16 μ g/ml), and the mixture was incubated with gp120 for 1 h. The amount of 39.13g bound to gp120 in the presence or absence of the test MAbs was determined.

Cell surface MAb and sCD4 binding. Infected H9 cells were washed twice in complete phosphate-buffered saline (PBS)–1% fetal calf serum–0.05% sodium azide (WB), resuspended at a concentration of 10^7 cells per ml in WB, and chilled on ice. MAbs or sCD4 were diluted in WB, and 100 μ l was mixed with 100 μ l of cells (10^6 cells) and incubated in a V-bottom microtiter plate for 30 min. Cells were washed with WB by centrifugation, and the bound MAb was detected with a 1/40 dilution of fluorescein isothiocyanate-conjugated anti-rat immunoglobulin G (IgG) or anti-human IgG (SeraLabs; Crawley, Sussex, United Kingdom) in WB. Bound sCD4 was detected with CD4-specific MAb L120 (MRC ADP Repository) and fluorescein isothiocyanate-conjugated anti-mouse IgG. After a 30-min incubation on ice with the conjugate, the cells were washed three times with WB and inactivated by resuspension in 500 μ l of 1% paraformaldehyde in PBS at 4°C overnight. Cells were analyzed on a FACStar, using Lysis software (Becton Dickinson). The results shown are the mean fluorescence intensities obtained for one experiment. However, all experiments were repeated three times.

Cassette vector construction and site-directed mutagenesis. The HXB2 insert of plasmid pHXB2gpt was transferred into a vector lacking *Xba*I sites by ligation into the *Nhe*I site of a pSPTBM20 vector (Boehringer Mannheim), from which the polylinker had previously been excised by double digestion with *Sma*I and *Eco*RV and self-ligation. A derivative, MCS, was constructed by introducing a silent site-directed mutation in the gp160 gene to generate an *Xba*I site and two nonsilent changes in the nonfunctional *vpu* gene to generate a *Nor*I site. Virus recovered upon transfection of the modified plasmid was able to infect H9 cells with the same infection kinetics as the parental HXB2. Infection of 10^6 H9 cells with viral doses of HXB2 and HXB2-MCS containing 200 pg of virion p24 resulted in soluble p24 production at 4, 6, 8, and 10 days postinfection of 7.6, 120.0, 390.0, and 510.0 ng/ml and 5.0, 185.0, 425.0, and 525.0 ng/ml, respectively. A deleted form of MCS made by double digestion of the construct with *Nhe*I and *Mam*I followed by Klenow fill-in of the *Nhe*I end and self-ligation. This digestion excised a 295-bp piece of the envelope gene including the V4, C4, and V5 domains (see Fig. 3), and the plasmid was shown to be noninfectious (data not shown).

Generation and cloning of wild-type and variant gp120 molecules. Single molecules of gp120 were amplified by a limit dilution method (31) using nested primers specific for the *vpu* region (sense) and for the gp41 molecule (antisense). Initial amplification was performed in a volume of 20 μ l with primers TCA TCA AGT TTC TCT AC/TC AAA GC (5' sense, outer; positions 5568 to 5590 (23a) and TCC CAC TCC ATC CAG GTC (3' antisense, outer; positions 7664 to 7647) (23a), using 30 cycles of polymerase chain reaction (PCR) amplification (94°C, 40 s; 50°C, 35 s; 72°C, 210 s) with

TABLE 1. Neutralization sensitivities of wt and variant viruses

Ligand	Epitope	Neutralization titer ($\mu\text{g/ml}$ or reciprocal dilution ^a)	
		wt	Variant
41.1	V3	0.4 ± 0.05	0.5 ± 0.06
10/54	V3	9.4 ± 1.8	8.4 ± 1.6
10/36e	V3	1.2 ± 0.4	0.8 ± 0.3
11/85b	V3	1.3 ± 0.5	1.1 ± 0.3
10/76b	V2	0.9 ± 0.2	1.2 ± 0.3
39.13g	CD4 b.s.	5.4 ± 0.8	NR ^b
sCD4		2.5 ± 0.4	NR
QC5 (selecting serum)		1/640	1/150

^a Reciprocal dilution is given for QC5.

^b NR, 90% reduction in p24 antigen production was not achieved for the variant virus with either 39.13g or sCD4. At 20 $\mu\text{g/ml}$, the variant was neutralized by 10 and 53% by 39.13g and sCD4, respectively.

16 ng of each primer, 200 μM each deoxynucleoside triphosphate (dNTP), 50 μM TMAC, 1 \times PFU of polymerase buffer 3, and 0.5 U of polymerase (Stratagene). To prevent degradation, the first-round product was frozen immediately upon completion of the reaction. One microliter of this first-round product was transferred to a second tube containing a volume of 100 μl with primers GTA GCA TTA GCG GCC GCA ATA ATA GCA ATA G (5' sense, *NotI* inner; positions 5635 to 5668) (23a) and GTT CTA GAG ATT TAT TAC TCC (3' antisense, *XbaI* inner; positions 7631 to 7611) (23a), using 25 cycles of amplification (94°C, 35 s; 55°C, 25 s; 72°C, 150 s) with 80 ng of each primer, 200 μM each dNTP, 50 μM TMAC, 1 \times PFU polymerase buffer 3, and 2.5 U of PFU polymerase. Ten microliters of the PCR product was visualized on an agarose gel, and the remainder was purified by a Gene-Clean (Bio 101, La Jolla Calif.) procedure. After digestion with *NotI* and *XbaI* (Promega Biotec), and PCR product was ligated into *NotI-XbaI*-digested pHXB2-MCS Δenv and cloned into *Escherichia coli* SURE (Stratagene). Colonies were screened for full-length clones by PCR and full-length clones were sequenced to confirm the insert genotype.

Site-directed mutation and cloning of gp120 molecules. Site-directed mutagenesis of the HXB2 envelope was performed by a modification of the method of Wolfs et al. (38), using PFU polymerase in place of *Taq* polymerase. Two 20- μl PCR reactions were set up as described above, with 25 ng of pHXB2gpt as the template, using either the 3' antisense *XbaI* primer and a sense primer encoding the desired 375 mutation or the 5' sense *NotI* inner primer and a second, antisense, 375 primer. The use of this relatively large amount of template DNA allowed us to reduce the number of cycles performed in the PCR and hence the frequency of polymerase-introduced error; the PCR was run for 10 cycles, using the temperature profile described above for the second round. The PCR products were gel purified by a Gene-Clean procedure, and the two products were pooled into a 100- μl PCR mix containing the 5' sense *NotI* inner primer and the 3' antisense *XbaI* primer; this mixture was reamplified for a further 10 cycles, using the same temperature profile as before. The final, full-length product was separated from the two shorter products on a 1.5% agarose gel, purified, cloned, and screened as described above. The presence of the desired change was verified by sequencing.

RESULTS

Selection procedure. Experiments were designed to monitor the kinetics of the appearance of HIV-1 escape mutants propagated in the presence of a HIV-1-positive human serum. The serum chosen, QC5 (16), neutralized HXB2 at a serum dilution of 1/640 and contained antibodies capable of blocking the gp120-sCD4 interaction (data not shown). Virus

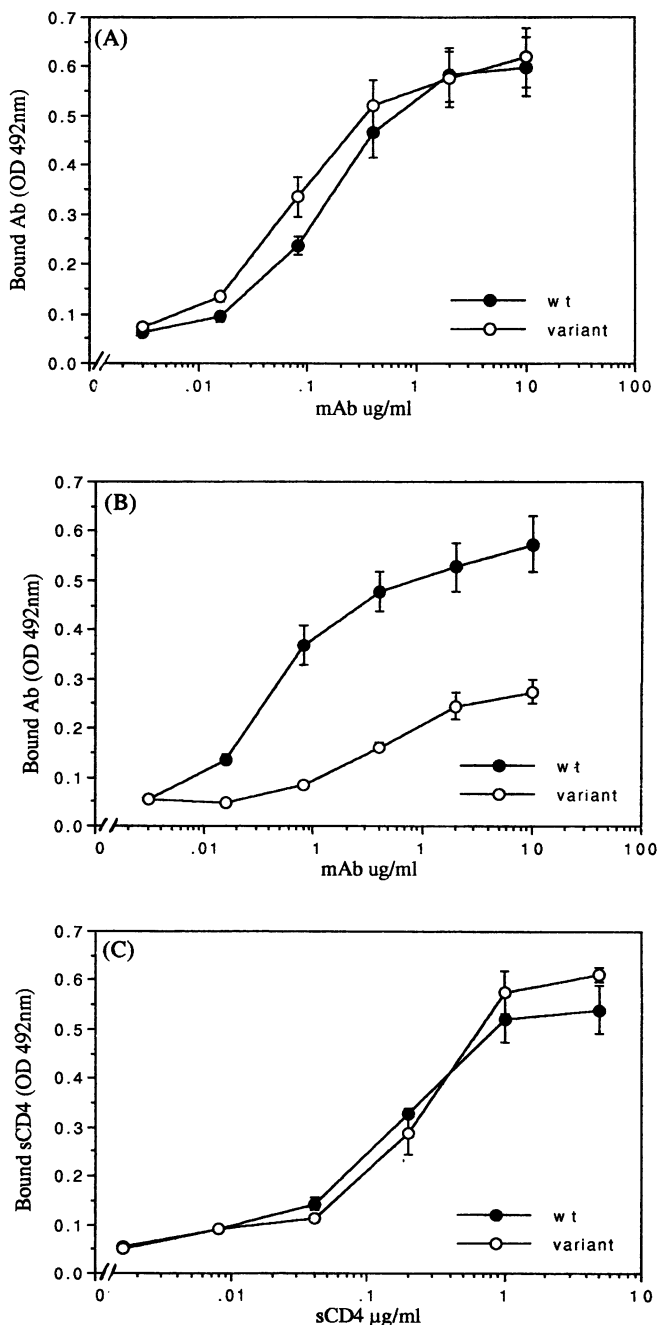


FIG. 1. Binding of MAb 41.1, mapping to the V3 loop (A), 39.13g, mapping to a discontinuous epitope overlapping that of the CD4 b.s. (B), and sCD4 (C) were analyzed for the ability to bind to NP-40-solubilized viral gp120 from wt and variant viral stocks. OD, optical density.

obtained by transfection of the HIV-1 molecular clone HXB2 into C8166 cells was allowed to infect the H9 cell line at multiplicities of infection of 0.1 and 0.01 for 2 h at 37°C. After viral adsorption, unbound virus was removed by washing and the cells were cultured in the presence of either a seronegative human serum (AB⁺) or serum QC5 at final dilutions of 1/40, 1/80, 1/160, and 1/320. Cultures were fed biweekly with the human sera and were monitored for both cytopathic effect and p24 antigen production.

After 2 weeks, virus was recovered as cell-free supernatant from the cultures containing the lowest serum dilutions (1/160 and 1/320). After 4 weeks of propagation, viral antigen (p24) was detected in the culture containing QC5 at a 1/80 dilution. Over a 7-week period, no evidence for viral replication was observed in the highest concentration of selecting serum (1/40). The virus-containing supernatants were tested for sensitivity to neutralization by the selecting serum. Virus rescued from the cultures containing QC5 at a 1/80 dilution was partially resistant to neutralization by QC5 (Table 1). However, virus obtained in the presence of the two lowest concentrations of QC5 was not resistant to neutralization by the selecting serum (data not shown).

This partially resistant virus was tested for its sensitivity to neutralization by sCD4 and a number of rat gp120 MAbs: 39.13g, binding to a discontinuous epitope overlapping with that of CD4 and capable of blocking the gp120-sCD4 interaction (3, 19); 10/54, 10/36e, 11/85b, and 41.1, binding to epitopes in the V3 loop (14); and 10/76b, binding to a linear epitope in the V2 loop (17a). The variant was resistant to neutralization both by MAb 39.13g and by sCD4 (Table 1). However, the variant was as susceptible as the parental virus to neutralization by all of the V2 and V3 MAbs (Table 1).

MAb recognition of variant gp120. We measured the ability of sCD4 and MAbs 39.13g and 41.1 to bind to nonionic detergent (NP-40)-solubilized wt and variant viral gp120 (at an input concentration of 50 ng/ml). The V3 MAb 41.1 bound to the variant and wt gp120 with comparable affinities (Fig. 1A). In contrast, MAb 39.13g showed a reduced binding affinity (10.6-fold) for the variant gp120 (Fig. 1B), in agreement with the reduced ability of the MAb to neutralize the variant. However, sCD4 bound to the variant gp120 with an affinity comparable to that of the wt (Fig. 1C), which was unexpected given the reduced ability of sCD4 to neutralize the variant (Table 1). We have previously reported differences in the abilities of both MAbs (14) and sCD4 (22) to bind to native virion gp120 and detergent-solubilized gp120. We therefore monitored the binding of MAbs and sCD4 to native envelope expressed on the surface of infected cells. sCD4 induces gp120 shedding from the cell surface at 37°C (23); therefore, all ligand titration curves were performed at 4°C to prevent this. In agreement with the ELISA data, MAb 39.13g (at a saturating concentration) bound less well to variant-infected H9 cells than to wt-infected cells, resulting in a mean fluorescence intensity of 50.2 versus 71.5, respectively (Fig. 2B). A saturating concentration of sCD4 (10 µg/ml) bound equivalently to wt-infected and variant-infected cells, with mean fluorescence intensities of 170 and 176, respectively (Fig. 2C). However, concentrations of sCD4 resulting in half-maximal binding (1.0 µg/ml) demonstrated a twofold reduction in binding to variant-infected cells compared with wt-infected cells, suggesting that the native variant envelope had a reduced binding affinity for sCD4 (Fig. 2C). V3 MAb 41.1 bound to wt-infected and variant-infected cells with similar affinities, suggesting that the level of gp120 cell surface expression was comparable in

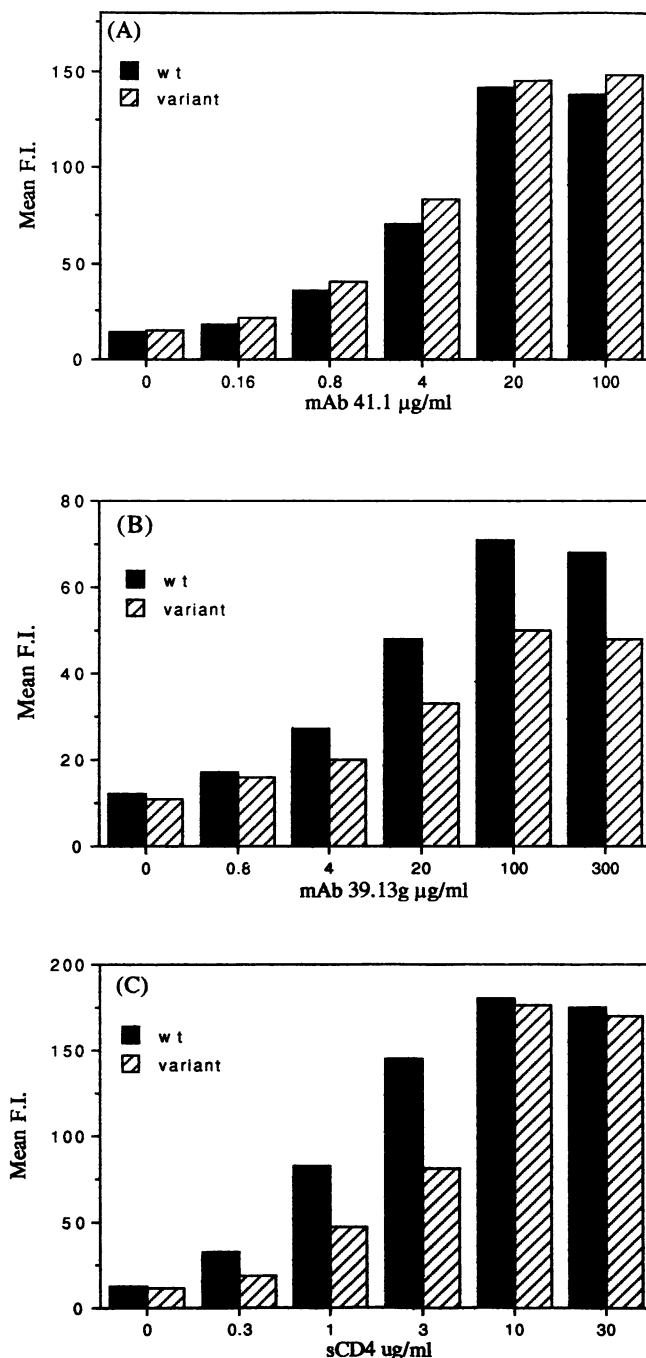


FIG. 2. Binding of MAbs and sCD4 to wt- and variant-infected cells. Increasing concentrations of MAbs 41.1, mapping to the V3 loop (A), 39.13g, mapping to a discontinuous epitope overlapping that of the CD4 b.s. (B), and sCD4 (C) were analyzed for the ability to bind to wt- and variant-infected H9 cells at 4°C. The mean fluorescence intensity (F.I.) from an individual experiment is shown.

the infected cultures (Fig. 2A). The reduced binding of sCD4 to the variant-infected cells compared with the wt-infected cells was not attributable to differential sCD4-induced gp120 shedding, since the concentration of extracellular soluble

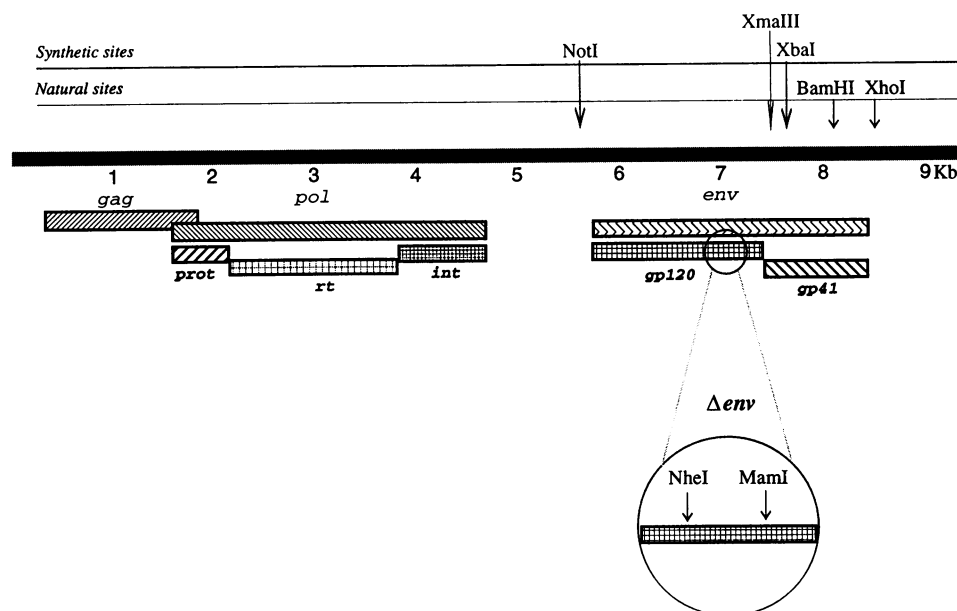


FIG. 3. pHXB2-MCS construct. The diagram shows the locations of the major genes and restriction sites used in this study. The Δenv construct is shown by the circle in which the *NheI* and *MspI* sites used for the deletion are noted. *prot*, protease; *rt*, reverse transcriptase; *int*, integrase.

gp120 was found to be identical for the wt- and variant-infected H9 cells (data not shown).

Sequence analysis of variant and site-directed mutagenesis. We have previously identified gp120 residues 113, 117, 256, 257, 262, 368, 370, and 421 as being involved in the binding of MAb 39.13g (19). These regions overlap with those reported to be important for sCD4 binding (25). We therefore sequenced the gp120 of both the variant and wt viruses and identified a single amino acid substitution at codon 375 (AGT→AAT, Ser→Asn; designated 375 S/N). To confirm that the gp120 mutation at 375 is responsible for the resistance of the variant to 39.13g neutralization, we cloned both the wt and variant gp120 genes into a viable HIV-1 cassette vector, pHXB2-MCS (Fig. 3). In addition, we generated a synthetic mutation at codon 375 by site-directed mutagenesis and also cloned this mutated gp120 into pHXB2-MCS. The viral stocks recovered from transfection with several clones, named HXB2-wt, HXB2-var, and HXB2-375 S/N, respectively, were tested both for their sensitivities to neutralization by the original selecting serum QC5 and a range of neutralizing MAbs and for their abilities to bind MAbs. HXB2-var (clone 23) and HXB2-375 S/N (clone 9) were both resistant to neutralization by QC5 and 39.13g but remained sensitive to neutralization by MAbs specific for the V2 and V3 domains (Table 2), confirming that the mutation at residue 375 is responsible for the neutralization-resistant phenotype. Additional clones of HXB2-var and HXB2-375 S/N demonstrated similar neutralization profiles (data not shown).

Neutralization and MAb binding to the 375 S/N site-directed mutant. There are a number of neutralizing human MAbs which block gp120-sCD4 binding and which have similar binding properties to a panel of site-directed gp120 mutants as 39.13g (19, 30, 33, 35). All of the human MAbs tested were able to cross-compete with 39.13g for gp120 binding (Fig. 4), suggesting that the epitopes recognized by the human MAbs may overlap that of 39.13g. We therefore investigated the

sensitivity of HXB2-375 S/N to neutralization by a panel of nine human MAbs. Both HXB2-var and HXB2-375 S/N were as sensitive as HXB2-wt to neutralization by the CD4 b.s. MAbs 559, 728, 654, 729, 588 (12), and 2.1h (35) but were resistant to neutralization by three additional MAbs, 1.5e (9), G13 (30), and 448 (12) (Table 2). MAbs 1.5e and G13 showed reduced binding indices for both the cloned variant and the 375 S/N site-directed mutant viral gp120, both by ELISA (Fig. 5) and by cell surface fluorescence-activated cell-sorting (FACS) analysis (data not shown), in good agreement with the observed resistance to neutralization. However, MAb 448, which demonstrated a reduced ability

TABLE 2. Neutralization sensitivities of HXB2-wt, HXB2-var, and HXB2-375 S/N^a

Ligand	Epitope	% Neutralization		
		HXB2-wt	HXB2-var	HXB2-375 S/N
559	CD4 b.s.	83 ± 14	43 ± 9	54 ± 10
448	CD4 b.s.	72 ± 6	2 ± 0.5	4 ± 0.3
728	CD4 b.s.	80 ± 17	59 ± 16	68 ± 13
654	CD4 b.s.	84 ± 18	61 ± 13	78 ± 18
729	CD4 b.s.	44 ± 9	54 ± 8	67 ± 11
588	CD4 b.s.	85 ± 14	87 ± 19	93 ± 20
G13	CD4 b.s.	95 ± 8	4 ± 0.3	3 ± 0.2
1.5e	CD4 b.s.	56 ± 7	3 ± 0.2	4 ± 0.3
2.1h	CD4 b.s.	67 ± 9	56 ± 7	62 ± 10
39.13g	CD4 b.s.	94 ± 14	10 ± 0.9	8 ± 0.7
11/85b	V3	96 ± 6	89 ± 14	92 ± 15
41.1	V3	98 ± 7	96 ± 6	98 ± 6
10/76b	V2	96 ± 8	94 ± 10	97 ± 9
sCD4		94 ± 7	37 ± 2	28 ± 3
QC5 (human serum)		94 ± 8	43 ± 6	38 ± 4

^a All antibodies and sCD4 were tested at a final concentration of 10.0 µg/ml with the exceptions of 559 (0.86 µg/ml), 728 (2.20 µg/ml), 654 (5.30 µg/ml), and 729 (1.94 µg/ml), which were tested at the highest concentrations feasible (in parentheses). The selecting serum QC5 was tested at a final dilution of 1/100.

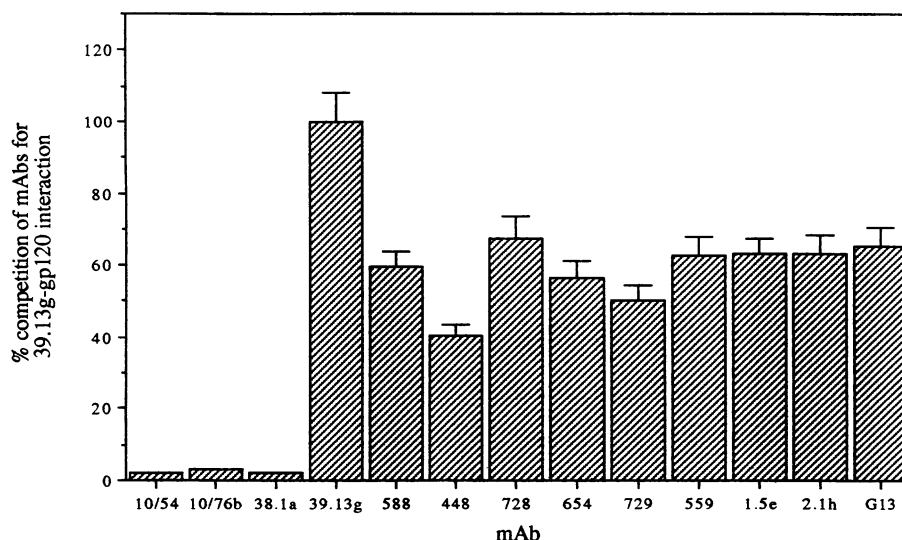


FIG. 4. Cross-competition analysis for MAb 39.13g binding to gp120. MAbs (at a saturating concentration of 5.0 μ g/ml) mapping to a linear V2 epitope (10/76b), to a linear C4 epitope (38.1a), to a linear V3 epitope (10/54), and to epitopes overlapping the CD4 b.s. (588, 448, 728, 654, 729, 559, 1.5e, 2.1h, and G13) were tested for their ability to compete with iodinated 39.13g for binding to gp120. The results represent means of triplicate wells and are from one experiment, but similar results were obtained in two separate experiments.

to neutralize both the variant and 375 S/N mutant viruses (Table 2), bound to detergent-solubilized gp120/160 with binding indices of 0.81 and 0.82, respectively. Furthermore, no detectable difference in the ability of MAb 448 to bind to the surface of wt- or variant-infected H9 cells was observed by FACS staining (data not shown). The remaining six human MAbs and three control MAbs, mapping to V2, V3, and C4, bound to the variant and 375 S/N site-directed mutant antigens with binding indices ranging from 0.8 to 1.20 (Fig. 5).

Since MAbs 39.13g, 1.5e, and G13 still bind to HXB2-375 S/N, though with reduced binding indices, we investigated the mechanism of neutralization resistance. Since all of these MAbs block in vitro the gp120-sCD4 interaction, they presumably neutralize by preventing virion attachment to CD4. We therefore tested the ability of a number of CD4 b.s. MAbs, together with control MAbs specific for the V2, V3, and C4 regions of gp120, to block sCD4 binding to NP-40-solubilized HXB2-wt, HXB2-var, and HXB2-375 S/N viral gp120 by ELISA. MAbs 39.13g, 1.5e, and G13 (5 μ g/ml) blocked sCD4 binding to HXB2-wt gp120 by 82, 83, and 79%, to HXB2-375 S/N mutant by 27, 29, and 20, and to HXB2-var by 24, 22, and 26%, respectively (Fig. 6). Since the gp120-sCD4 binding assay was performed with NP-40-solubilized viral gp120, it is not surprising that MAb 448 inhibited sCD4 binding to wt and variant gp120 equivalently (Fig. 6).

Since the 375 S/N mutation was originally selected for by an HIV-1-positive human serum, we investigated the sensitivity of the variant to neutralization by a panel of sera from HIV-1-infected individuals classified in CDC stages II and III (Fig. 7). Eleven of 21 sera showed a reduced neutralization titer for the variant, with a median neutralization titer of 1/20, compared with 1/40 for HXB2-wt ($P = 0.0092$, two-tailed Wilcoxon rank test).

DISCUSSION

We have selected an HIV-1 variant which can replicate in the presence of a neutralizing human serum (Table 1).

Sequencing of the gp120 genes from both the variant and the wt virus identified a single amino acid change in the C3 region of gp120, at residue 375 from Ser to Asn. Characterization of this mutation, both as a cloned variant gp120 (HXB2-var) and as a site-directed mutant (HXB2-375 S/N), demonstrated that the mutation conferred resistance to neutralization by MAbs 39.13g (3), 1.5e (9), G13 (30), and 448 (12), binding to epitopes overlapping the CD4 b.s., and by sCD4 (Table 2). In agreement with the reduced sensitivity to neutralization, MAbs 39.13g, 1.5e, and G13 demonstrated reduced binding to NP-40-solubilized HXB2-var and HXB2-375 S/N viral gp120 with binding indices of 0.48, 0.42, and 0.46 and of 0.42, 0.45, and 0.43, respectively (Fig. 5). However, MAb 448 bound to the cloned variant and mutant proteins with binding indices of 0.81 and 0.82. The relatively modest change in binding index observed with both MAb 448 and sCD4 for the variant and the 375 S/N mutant, despite the reduced neutralization sensitivity, suggests that studying the binding of ligands to detergent-solubilized viral antigen may not accurately predict the behavior of such ligands with the native protein. We have previously reported a difference in the ability of sCD4 to bind to soluble gp120 and native intact virion gp120 (22), suggesting that the tertiary and/or quaternary structure of native oligomeric gp120/41 influences the binding affinity of sCD4.

MAbs 39.13g, 1.5e, and G13 were less efficient at blocking sCD4 binding to solubilized HXB2-var and HXB2-375 S/N viral gp120 than to HXB2-wt gp120 (Fig. 6), suggesting that the efficiency of neutralization by CD4 b.s. antibodies may be governed by the affinity of the antibody for the envelope glycoprotein. By using a panel of site-directed gp120 mutants, the epitopes recognized by the CD4 b.s. MAbs, with the exception of 728 and 654, have been mapped (19, 30, 33, 35) to five discontinuous regions including amino acids 113 to 117 in C1, 256 to 262 in C2, 368 to 370 in C3, 421 to 427 in C4, 457 in C4, and 475 to 477 in C5. The regions of gp120 recognized by the MAbs differ slightly, but most depend on a core of amino acids including Asp-368, Glu-370, Thr-257, and Lys-421. There is no obvious pattern from the mapping

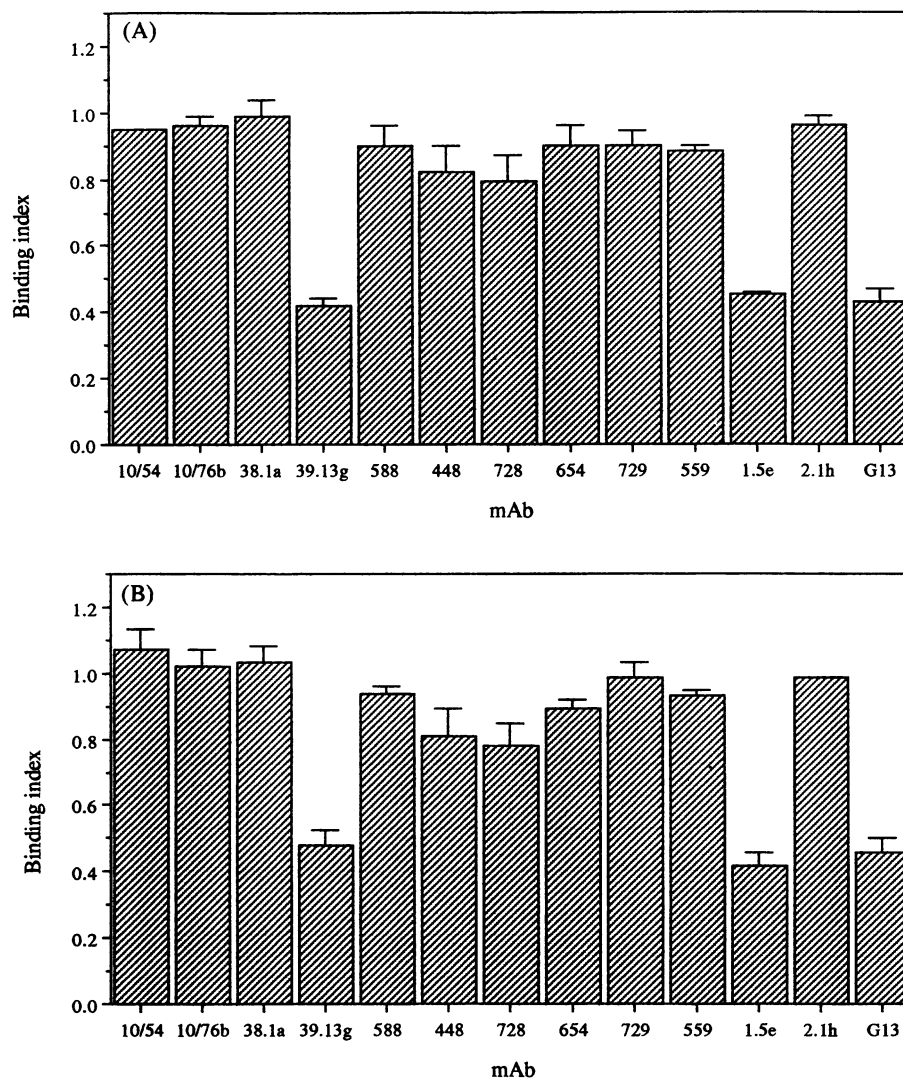


FIG. 5. Binding of MAbs to wt, variant, and 375 S/N mutant gp120/160. MAbs (at a saturating concentration of 5.0 μ g/ml) mapping to a linear V2 epitope (10/76b), to a linear C4 epitope (38.1a), to a linear V3 epitope (10/54), and to epitopes overlapping the CD4 b.s. (39.13g, 588, 448, 728, 654, 729, 559, 1.5e, 2.1h, and G13) were compared for the ability to bind to NP-40-solubilized gp120/160 from HXB2-375 S/N (A) and HXB2-var (B) with HXB2-wt. The results are expressed as the ratio of MAb (optical density) bound to the variant or site-directed mutant relative to the wt and are termed the relative binding index.

data to explain why MAbs 39.13g, 1.5e, G13, and 448 are unable to neutralize the escape mutant whereas MAbs 559, 728, 654, 729, 588, and 2.1h neutralize the mutant as well as the wt virus. Amino acids from four of these regions (Thr-257, Asp-368, Glu-370, Trp-427, and Asp-457) have been implicated in CD4 binding (25). Thus, a number of amino acid residue changes significantly affect MAb binding without affecting sCD4 recognition, suggesting that viral escape from antibody neutralization may occur without affecting the ability of the virus to bind to its receptor (19). However, the 375 S/N variant was also resistant to neutralization by sCD4 (Tables 1 and 2). sCD4 demonstrated a reduced binding affinity for native envelope but not for detergent-solubilized gp120 (Fig. 1 and 2). Olshevsky and colleagues (25) reported that mutation of residues 368 and 370 abrogated sCD4 binding in the presence or absence of nonionic detergents; however, residue 375 was not studied by these authors.

Reitz and colleagues (27) reported on a serum-selected

escape mutant which had a single change in gp41 at residue 582 (Ala-Thr, or A/T), which exhibited a decreased susceptibility to neutralization by approximately one-third of human sera tested. Since antibodies to linear epitopes encompassing residue 582 were not neutralizing, Wilson and colleagues (37) hypothesized that a neutralization epitope(s) may be affected by the change in gp41. More recently, we (12b) and others (31a) have reported that the 582 A/T mutant is insensitive to neutralization by a number of CD4 b.s. MAbs, including F105 (26), 1.5e (9), and 39.13g (3). The 582 A/T mutant appears to bind the MAbs with an affinity equivalent to that of the wt. This is in contrast to the 375 S/N mutant, which has a reduced affinity for MAbs 39.13g, G13, and 1.5e (Fig. 5). This finding suggests that escape from CD4 b.s. MAbs may occur by one of at least two mechanisms, either by a mutation which directly reduces the affinity of the MAb for gp120 (Fig. 5) or by a mutation which indirectly affects events after MAb binding. Alternatively, a small

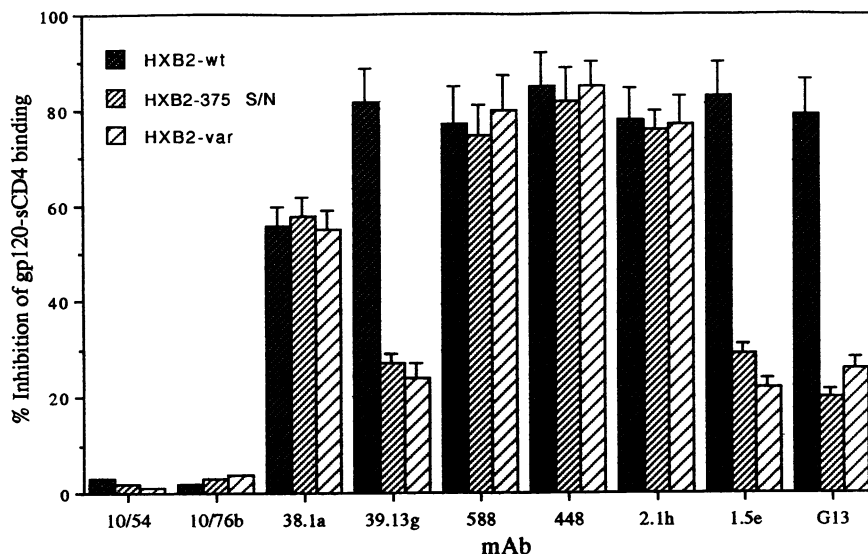


FIG. 6. Abilities of MABs to inhibit the interaction of HXB2-wt, HXB2-var, and HXB2-375 S/N gp120/160 with sCD4. MABs (at a saturating concentration of 5.0 μ g/ml) mapping to a linear V2 epitope (10/76b), to a linear C4 epitope (38.1a), to a linear V3 epitope (10/54), and to epitopes overlapping the CD4 b.s. (39.13g, 588, 448, 1.5e, 2.1h, and G13) were tested for the ability to inhibit sCD4 (1.0 μ g/ml) binding to NP-40-solubilized gp120/160 (50 ng/ml) from HXB2-wt, HXB2-var, and HXB2-375 S/N viral stocks.

reduction in the affinity of mutant 582 A/T for the MABs or of mutant 375 S/N for MAB 448, not measurable by current assays, may be sufficient to account for the differential neutralization observed (32).

The 375 S/N mutant replicates in CD4⁺ cells as efficiently as the wt does, as assessed by measuring the infectivity-to-particle ratio (TCID₅₀ virion p24) (data not shown) (17). This finding is in agreement with the ability of sCD4 and sCD4-IgG neutralization-resistant variants to replicate as well as the neutralization-sensitive parental virus does (12a, 13). This observation is consistent with reports that replication-competent viruses can exhibit a wide range of CD4-binding affinities and may be explained by the greater cooperativity

of envelope-receptor interactions during virus-cell or cell-cell fusion compared with the interaction of soluble CD4 and gp120 (32).

The ability of a polyclonal serum to select for a mutation in C3 which affects the sensitivity to neutralization both by sCD4 and CD4 b.s. MABs confirms that antibodies binding to the CD4 b.s. contribute to the group-specific neutralization response (21, 32). However, the 375 S/N mutant was only partially resistant to 11 of 21 human sera tested (Fig. 7), suggesting that either the cross-neutralizing activity may be attributed to an epitope(s) in addition to the CD4 b.s. or the CD4 b.s. induces a complex antibody response involving many discontinuous overlapping epitopes. The latter sugges-

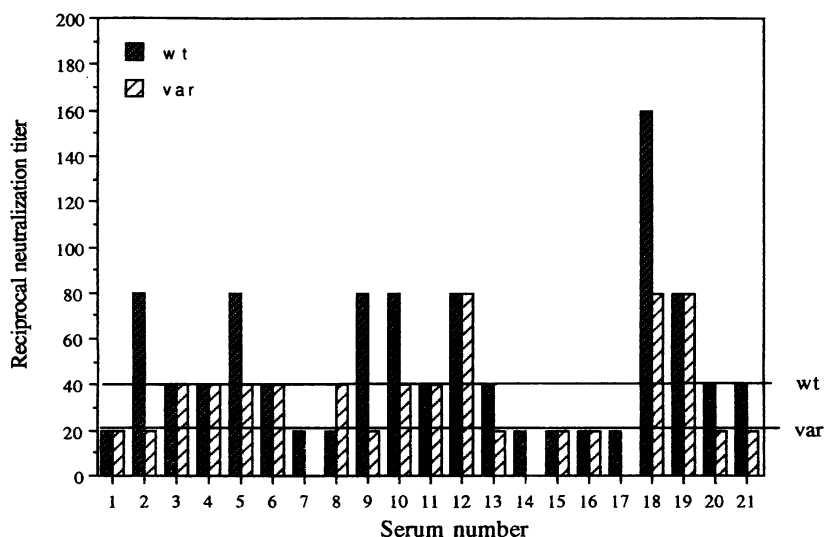


FIG. 7. Sensitivities of wt and variant viruses to neutralization by sera from HIV-1-infected individuals. Reciprocal neutralization titers of 21 human sera for wt and variant viruses are shown. Average titers (horizontal lines) were 1/40 for the wt and 1/20 for the variant (Wilcoxon rank test, $P = 0.009$).

tion is supported by the observation that three of nine human MAbs mapping to the CD4 b.s. failed to neutralize the 375 S/N mutant (Table 2). The subdivision of this panel of CD4 b.s. MAbs into groups based upon their ability to neutralize 375 S/N suggests that current methods of epitope mapping, recognition of synthetic or site-directed gp120 mutants, and cross-competition assays are insufficiently sensitive to define MAb epitopes completely. These data suggest that single amino acid changes resulting in resistance to one selecting agent may not lead to complete resistance to a polyclonal mixture of antibodies present in a heterologous serum.

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